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The possible physiological significance of the rates of formation and decomposition of enzyme-substrate complexes and compounds is mentioned. Then the potentialities of the stopped-flow methods with optical and chemical sampling are discussed briefly. They are illustrated with examples of information gained about the kinetic behaviour of some selected enzyme-substrate compounds.

Die mögliche physiologische Bedeutung der Bildungs- und Zerfallsgeschwindigkeiten von Enzym-Substratkomplexen und -verbindungen wird angedeutet. Dann wird die Leistungsfähigkeit der „stopped-flow"-Methoden mit optischer und chemischer Auswertung kurz diskutiert. Es folgen einige Beispiele für Informationen, die über das kinetische Verhalten einiger ausgewählter Enzym-Substratverbindungen gewonnen wurden.
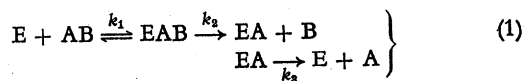
Several aspects of the study of transients in enzyme kinetics will be discussed in this article. The topics selected will, however, be only a small portion of the whole subject, which complements and extends the information obtained from the now classical steady state approach. First it is of interest to consider the possible physiological significance of the rates of formation and decomposition of enzyme-substrate complexes and compounds. Secondly the potentialities of the stopped-flow methods with optical and chemical sampling will be discussed briefly and they will be illustrated with examples of information gained about the kinetic behaviour of some selected enzyme-substrate compounds.

It is quite clear the reactions between enzymes and their substrates proceed via a number of distinct steps.

The enzymes to be discussed in the present paper do not have any prosthetic groups. One can demonstrate that the initial combination between enzyme and substrate to form the Michaelis complex is a very fast, largely diffusion controlled reaction. Subsequent to this diffusion controlled adsorption of the substrate onto the specificity site of the enzyme there occur chemical reactions between the catalytic groups on the enzyme and the substrate and also very likely structural changes of the enzyme itself. In some cases chemical compounds are formed between the enzyme and some part of the substrate, while in other cases less well defined intermediates are formed which decompose to free enzyme and product. Kinetic investigations on the formation and decomposition of such intermediates under a variety of conditions are essential for the elucidation of the chemical mechanisms involved in their reactions. The rates of intermediate steps in enzyme reactions can however also be of great importance for the explanation of physiological control phenomena without any reference to reaction mechanisms. In many systems enzymes occur locally at such high concentrations – at reagent concentrations – that some rapid transitions from one state to another are not controlled by the slow reaction of the enzyme but by an individual step such as the physical or chemical combination with the substrate of the release of a combined product. Examples of such control by transients may well be found in muscle, nerve or respiratory reactions with rapid "on" and "off" transitions.

The Approach to the Steady State

The study of enzyme reactions can now be divided into three time scales: (1) classical methods, seconds to hours; (2) flow methods, milliseconds to seconds; (3) relaxation methods, microseconds to milliseconds. Here we are concerned with the application of flow mixing methods for the recognition and characterization of steps in enzyme systems where the enzyme itself has – as yet – not revealed any spectral changes which can be used for transient studies. In such systems the disappearance of substrate and/or the appearance of products has to be monitored during the pre-steady state phase of the reaction. In some cases the substrate or product can be monitored directly trough a colour change or indirectly through indicators, if a p_H change occurs as a consequence of the reaction. The stopped-flow apparatus for observing spectral changes during the reaction (Gibson and Milnes [7]) has proved itself useful for the study of hydrolytic enzymes (Gutfreund [8]). As an example the trypsin catalysed hydrolysis of benzoyl L-arginine ethyl ester (BAEE) can be recorded from about 3 milliseconds after mixing in an apparatus with a 1 cm optical pathway and from about 1 millisecond with a 0.2 cm pathway. The steady state rate of acid production is reached within the time of resolu-

tion. The first order constants must be $> 300 \ sec^{-1}$, while the steady state turnover is 15 sec^{-1}. Although such limiting values are already of interest, real values would be of greater importance for the interpretation of the type of mechanism illustrated by the following general scheme:

$$E + AB \underset{}{\overset{k_1}{\rightleftharpoons}} EAB \overset{k_2}{\longrightarrow} EA + B \left.\begin{array}{l} \\ \\ \end{array}\right\} \quad (1)$$
$$EA \underset{k_3}{\longrightarrow} E + A$$

The steps characterized by k_2 and k_3 are reversible but due to the low concentrations of B and A under the condition of the experiments the reversal need not be taken into consideration, that is k_{-2} and k_{-3} are neglected in the kinetic equations. If k_2 and k_3 are of comparable magnitude or $k_2 \gg k_3$, at high concentrations of AB the rate of attainment of the steady state is a function of k_2 and k_3 (Gutfreund [8]). Improvements in the time resolution of the stopped-flow methods will be achieved but it appears doubtful whether a procedure involving mixing will ever give a resolution below 0.3 millisecond. It is, however, possible to use competitive inhibitors to slow down some of the first order steps. This is a more promising approach than the slowing down of the reaction by performing measurements at a sub-optimal p_H since the latter procedure would remove a valuable variable for the characterisation of the reaction steps. With the simple assumption that the concentration of substrate [AB] and the competitive inhibitor [I] are sufficiently large so that all the enzyme present is rapidly converted to EAB and EI, one can derive the following expression for [EAB] in terms of the total enzyme concentration [E], prior to any significant conversion of [EAB] to [EA]:

$$[EAB] = \frac{[E]_0}{1 + \dfrac{K_m [I]}{K_i [AB]}}$$

In the absence of I the initial rate of production of EA is

$$\frac{d[EA]}{dt} = k_2[EAB] = k_2\{[E]_0 - [EA]\}$$

and in the presence of I

$$\frac{d[EA]}{dt} = k_2[EAB] = \frac{k_2}{1 + \dfrac{K_m [I]}{K_i [AB]}} \{[E]_0 - [EA]\}$$

If $k_2 \gg k_3$ the expression will hold over most of the first order conversion of EAB to EA.

The apparent values for k_2 obtained from transient kinetic measurements will thus be dependent on [I] and the real value can be calculated from extrapolation of the data to [I] → 0. This procedure is applicable both to the study of the initial rate of appearance of A or B in the two methods available for the evaluation of k_2 (Gutfreund [8]) and should extend the time scale of rate constants which can be measured by flow techniques.

Chemical Sampling

More general methods are required for the study of transients in reactions which can be followed directly by the continuous monitoring of the change of some physical quantity. For this purpose we have investigated the potentialities of chemical quenching and subsequent analysis of reaction mixtures. We first used a simple modification of an apparatus whose characteristics are well known, namely the original Gibson [6] stopped-flow apparatus. We substituted a motor drive for the manual pushing of the two syringes. With the motor running continuously the syringe movement is activated by microswitch operation of a magnetic clutch and a magnetic brake. This device has proved satisfactory with a flow of 7 ml/sec of reaction mixture through capillary tubes of 0.1 cm diameter. The length of the capillary, which determines the volume of the reaction tube, can be varied to alter the time elapsed between mixing enzyme and substrate and quenching in an acid solution. Chemical, enzymic and radioactivity analyses on the quenched reaction mixture can be used for the interpretation of the results. Chemical and enzymic tests showed that quenching can be achieved within 2 milliseconds or less (Barman and Gutfreund [2]). The ionic reaction of quenching by p_H change is faster than any of the steps examined but efficient mixing of the jet entering the quenching solution from the capillary is critical. The acidity of the quenching solution will also determine the speed with which the "quenching p_H" is reached.

Two enzyme systems have been studied so far with this method: bacterial alkaline phosphatase and trypsin. Alkaline phosphatase from various animal and bacterial sources will react with inorganic phosphate in slightly acidic solutions resulting in the phosphorylation of a serine group of the enzyme (Schwartz and Lipmann [9]; Engstrom [4, 5]). In neutral or alkaline solutions this phosphoryl enzyme decomposes rapidly to free enzyme and orthophosphate. Rapid reaction techniques are required to investigate the kinetics of the steps involved and to discover whether they are compatible with the steady state kinetic behaviour of the overall hydrolysis reaction catalysed by this enzyme. In this way it can be shown whether the phosphoryl enzyme is an obligatory intermediate in the reaction. Aldridge, Barman and Gutfreund [1] studied the rate of hydrolysis of the phosphoryl enzyme (E. coli alkaline phosphatase) by mixing ^{32}P labelled enzyme in acidic solution with buffers of different neutral and alkaline p_H and then quenching in acid again in the rapid flow quenching apparatus. The phosphatase precipitated with perchlorate was freed from inorganic phosphate and the protein bound ^{32}P counted to obtain data on the amount of phosphoryl enzyme remaining after different time intervals. At p_H 8.4 a first order plot was obtained giving a rate constant of 115 sec^{-1} at 20°. The overall turnover (k_0) of a number of

phosphate esters is approximately 30 sec^{-1} and the expression

$$k_2 = \frac{k_0 k_3}{k_3 - k_0} = 41 \text{ sec}^{-1}$$

can be used to complete the data if the reaction is interpreted in terms of the above scheme equation (1) with EA representing the phosphoryl enzyme. With this ratio of rate constants for the phosphorylation and dephosphorylation of the enzyme approximately 30% of the enzyme should be phosphorylated during steady state hydrolysis. The ratio of constants is critically p_H dependent.

The other example of an enzyme reaction studied by the flow-quenching technique is the trypsin catalysed hydrolysis of benzoyl L-arginine ethyl ester already discussed in a different context. There is general agreement on the kinetic sequence of events: initial adsorption of substrate, chemical interaction between enzyme and substrate, decomposition to free enzyme and products. There is, however, an interesting controversy about the nature of the chemical intermediate in trypsin, chymotrypsin and related enzyme reactions (Bernhard and Gutfreund [10]). With such substrates as nitrophenyl esters definite chemical intermediates in the form of acyl-enzymes have been demonstrated. Benders [3] studies gave similar results under a variety of conditions, but it had yet to be shown that alkyl esters and amides of amino acids reacted via acyl-enzyme compounds.

We have now started to examine the reactions of trypsin with its specific substrates (arginine derivatives) in the quenching apparatus (Barman and Gutfreund, to be published). It was stated above that the hydrolysis of BAEE catalysed by trypsin reaches its steady state within 3 millisec. If the enzyme is acylated during this short time period, one mole of alcohol per mole of enzyme must be liberated rapidly compared with the slow turnover which produces acid and alcohol with a half time of 50 millisec. The results obtained so far are somewhat complicated by the aggregation of the enzyme at the high concentrations used. In spite of sensitive alcohol analyses with alcohol dehydrogenase the trypsin concentrations in the reaction mixture were necessarily 10–100 μM. There is, however, little doubt that one mole of alcohol per mole of active enzyme is liberated at least 20 times faster than the steady state production, which indicates rapid acylation of the enzyme. The use of competitive inhibitors, as indicated above, should provide the facility to determine the rate constant for the initial liberation of alcohol.

Conclusions

The hydrolysis of esters is a reaction used as a good tool for the exploration of enzyme reactions because their chemistry is well understood. Amides and peptides, the natural substrates for proteolytic enzymes react much more slowly and acylation rather than the hydrolysis of the enzyme-acyl compounds will be rate deter-

mining. In the hydrolysis of phosphate esters the phosphorylation is rate determining and in experiments performed in collaboration with Prof. Wallenfels and Dr. Malhotra we have shown that in the galactosidase catalysed hydrolyses a similar step, rather than the breakdown of the enzyme-substrate compound is rate determining. This is the most efficient way of dealing with hydrolysis reactions the slow hydrolysis of enzyme-substrate compounds could, in that process, be regarded as a waste of time. In other enzymic processes, however, the enzyme substrate compound may play an important role and in such cases a different ratio of rate constants may result in the rapid formation of relatively stable enzyme-substrate intermediates.

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